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2.	Patent application number *(The Patent Office will fill in this part)*	0223449.0 09 OCT 2002
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	NOVARTIS FORSCHUNGSSTIFTUNG, ZWEIGNIEDERLASSUNG FRIEDRICH MIESCHER INSTITUTE FOR BIOMEDICAL RESEARCH, MAULBEERSTRASSE 66, CH-4058 BASEL SWITZERLAND
	Patent ADP number *(if you know it)*	
	If the applicant is a corporate body, give the country/state of its incorporation	SWITZERLAND
4.	Title of invention	Modulators of C-mannosyltransferase
5.	Name of your agent *(If you have one)*	Novartis Pharmaceuticals UK Ltd
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	Patents and Trademarks Wimblehurst Road HORSHAM West Sussex RH12 5AB
	Patents ADP number *(if you know it)*	ADP No. 07185220001

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MODULATORS OF C-MANNOSYLTRANSFERASE

The current invention relates to protein modifications, in particular to glycosylated proteins and to the enzyme that catalyzes a particular form of glycosylation, C-mannosylation.

BACKGROUND OF THE INVENTION

Glycosylation is one of the most abundant and widespread modifications of proteins and is increasingly being recognized to play an important role in a variety of biological processes. Recent examples are the modulation of Notch signaling by O-fucosylation (Moloney, D.J. et al., 2000, Nature, 406: 369-375; Brückner, K. et al., 2000, Nature, 406: 411-415), and the function of glucosylation in quality control of protein folding (Ellgaard, L. & Helenius, A., 2001, Curr. Opin. Cell Biol., 13: 431-437). As a consequence, deficiencies in glycoconjugate metabolism are now known to be associated with various pathological situations in humans. For example, Leucocyte Adhesion Deficiency II was shown to be caused by a molecular defect in GDP-fucose transport (Luhn, K. et al., 2001, Nat. Genet., 28: 69-72).

The best-studied forms of protein glycosylation, N- and O-glycosylation, have been known for approximately forty years. In these cases glycans are attached to the protein via an amide or hydroxyl group of an amino acid side chain, respectively. More recently, Hofsteenge and collaborators reported a new protein-carbohydrate linkage, the attachment of an alpha-mannopyranosyl residue to the C-2 atom of tryptophan in human RNase 2 (Hofsteenge, J. et al., 1994, Biochemistry, 33: 13524-13530; de Beer, T. et al., 1995, Biochemistry, 34: 11785-11789). This glycoconjugate does not contain the typical N- or O-glycosidic linkage but rather a C-C bond. The modification reaction is catalyzed by a microsomal C-mannosyltransferase (CMT), which uses dolichyl-phosphate-

compartment, with the isolation of minute amounts of modified material for analysis and with variability of results from cell to cell or cell-type to cell-type, leading generally to problems with reproducibility of the assay. There remains therefore a need for assays, in particular cell-based assays, amenable to high throughput formats to allow the identification of CMT as well as modulators of its activity and this invention meets that need.

SUMMARY OF THE INVENTION

The present invention provides methods for assaying for C-mannosyltransferase (CMT) activity, comprising the steps of: i) providing CMT and a CMT substrate, preferably in a cell, ii) providing conditions conducive to forming a C-mannosylated CMT substrate by action of the CMT on the CMT substrate; iii) immobilizing the C-mannosylated CMT substrate and (iv) detecting the C-mannosylated CMT substrate. The methods are particularly useful for screening for agents that modulate CMT activity.

Thus, in another aspect of the invention, methods of identifying agents effective in modulating C-mannosyltransferase (CMT) are provided. The methods comprise the steps of: i) contacting a cell comprising CMT with an agent; ii) detecting CMT activity; and iii) determining the agent-induced modulation in the CMT activity relative to when said agent is absent. Potential inhibitors of CMT activity as well as activators can be identified using these methods.

In a preferred embodiment, the screening methods of the invention are cell-based methods where a cell surface bound CMT substrate is expressed in the cell and C-mannosylation of the substrate is detected at the cell surface released from the cell surface after cleavage with a protease, for example. In this aspect of the invention, the C-mannosylated substrate will typically be presented as a fusion protein. The C-mannosylated substrate can be detected using a variety of means, such as with antibodies, detectable labels or tags.

The expression of CMT activity in a variety of cells has been studied and discussed in the scientific literature, including cells of insect, nematodes, amphibians, birds and mammals. Any of these cell and tissue sources or others yet to be discovered may be used as a source of CMT. For some purposes, the CMT will often be obtained from a cell line, such as HEK 293, COS7, CHO or NIH3T3 cells.

In one embodiment of the invention, CMT activity is assayed in vitro after the preparation of a cell extract. Methods for the preparation of cell extracts are known in the art (for example, see Scopes, 1987, Protein Purification: Principles and Practice, Second Edition, Springer-Verlag, N.Y.). Liver microsomes can also be used as the source of CMT as described previously (Doucey, M.-A. et al., 1998). Alternatively, a cell extract can also be prepared merely by lysing a cell sample to release CMT without further sample preparation.

Regardless of the origin of the CMT, the CMT sample (or an aliquot thereof) is assayed in a reaction mixture comprising a CMT substrate under conditions allowing C-mannosylation of the substrate. For, in vitro assays, this may mean incubating the reaction mixture in a buffer at physiological pH and at 37°C. Alternatively, the reaction may take place in a cell comprising the CMT substrate and CMT. The particular CMT substrate chosen in each case may vary depending on the type or origin of the CMT activity for which one is testing, or the type of detection method employed but will typically be a peptide or polypeptide comprising the consensus sequence WXXW.

The CMT substrate is designed to allow immobilization of the C-mannosylated CMT substrate to a solid phase (which includes a cell surface) or solid matrices. The CMT substrate is immobilized on a solid support in a manner such that C-mannosylation can be detected if the enzyme CMT is present in the sample (or cell). Immobilization can be achieved by any manner provided that detection is

the aminopeptidase N transmembrane domain. For example, the CMT activity assay is exemplified below (see Example 1) using a membrane bound CMT substrate construct. The construct encodes a fusion protein having an N-terminal domain comprising the cytoplasmic tail, transmembrane and stalk regions of human aminopeptidase N (APN) followed in N-terminal to C-terminal order, by a tobacco etch virus (TEV) protease site, glutathione S-transferase, a thrombin cleavage site and the CMT substrate, AWAQWA, or multiple copies thereof, expressed under the control of the CMV promoter. In some embodiments, the presence of multiple copies of the CMT substrate is particularly desirable, for example, when a reporter is immobilized and detected, or when it is desired to saturate the CMT with substrate so that activators can be found. As is apparent to one of ordinary skill in the art, variations in the design of the fusion protein are within the scope of the invention. In some embodiments, where anchorage to a cell membrane is not desired, it is also possible to use a polypeptide expression cassette additionally containing a signal sequence that causes the protein to be secreted into the medium.

In another embodiment, the CMT substrate can be linked to a reporter molecule. The reporter molecule (i.e., a signal generating molecule) can be any molecule capable of providing a detectable change. Such reporter molecules include fluorescent moieties (e.g., fluorescent proteins or chemical fluorescent labels), radioactive moieties, phosphorescent moieties, antigens, reporter enzymes and the like. Preferably, the reporter molecule is a reporter enzyme whose activity brings about a detectable change. Such reporter enzymes include, but are not limited to, the following: beta-galactosidase, glucosidases, chloramphenicol acetyltransferase (CAT), glucoronidases, luciferase, peroxidases, phosphatases, oxidoreductases, dehydrogenases, transferases, isomerases, kinases, reductases, deaminases, catalases and urease.

In selecting a reporter molecule to be used in the methods of the inventions, it is imperative that the reporter molecule is not subject to inactivation by any agent in

for C-mannosylated substrate. Such an antibody would not recognize the non-C-mannosylated form.

C-mannosylated substrates exhibited at the cell surface can be recognized using specific labelled antibodies and identified using FACS analysis, as is described in Example 1 below. Alternatively, if the fusion protein comprising the CMT substrate further comprises one or more protease cleavage sites flanking the CMT substrate, the CMT substrate can be isolated and C-mannosylation identified by mass spectrometry, as is described below in Example 2. Thus, it will be apparent to one of ordinary skill in the art that numerous variations exist for detecting C-mannosylation of a CMT substrate, for example, using an antibody or labelled moiety, such as the use of ^{3}H - mannose present in the buffer or cell. In addition, it will be apparent that the assays are easily amenable to high through-put technologies using robotics and automated processes. The methods are particularly useful for screening for agents that modulate CMT activity.

Screening Assays

In another aspect of the invention, methods of identifying agents effective in modulating C-mannosyltransferase (CMT) are provided. The methods comprise the steps of: i) contacting CMT (or a functional equivalent thereof) in a cell with an agent; ii) detecting CMT activity; and iii) determining the agent-induced modulation in the CMT activity relative to when said agent is absent. Potential inhibitors of CMT activity as well as activators can be identified using these methods. Thus, activators and inhibitors are referred to collectively herein as modulators and preferably influence CMT activity directly (i.e., not inhibit the DPM pathway).

anchored to the membrane by means of a GPI anchor. The protein inhibits the formation of the membrane attack complex of the complement system, by associating with C9, preventing its incorporation into C5b-8. This protects cells from complement-mediated lysis (Morgan, B.P. & Harris, C.L., 1999, Complement regulatory proteins, Academic Press, San Diego). However, in this aspect of the invention, CD59 merely serves its function as a marker for DPM synthesis. Other suitable markers will be apparent to those of ordinary skill in the art. If CD59 synthesis (or similar GPI anchor) were decreased, the methods of the invention would thereby indicate the presence of a modulator of GPI anchor synthesizing enzymes, which also provides useful information as lack of GPI is often associated with serious disease. Thus, the methods of the invention can also be used to identify modulators of GPI anchor synthesizihng enzymes.

The screening system is preferably used to screen agents that may be present in small molecule libraries, peptide libraries, phage display libraries or natural product libraries. Agent may be inorganic or organic, for example, an antibiotic or antibody. For ease of administration, the agent is preferably a small molecule.

Kits useful for screening such agents may also be prepared in accordance with the invention, and will comprise essentially CMT or a functional equivalent thereof useful for screening, and instructions. Typically the CMT will be provided together with one or more of a CMT substrate, a means for detecting CMT activity and at least one agent (putative agent).

CMT for use in kits according to the invention may be provided in the form of a protein, for example in solution, suspension or lyophilised, or in the form of a nucleic acid sequence permitting the production of CMT or a functional equivalent thereof in an expression system, optionally in situ.

Also provided are methods of identifying agents effective in modulating C-mannosyltransferase (CMT), said method comprising the steps of: (a) providing a

Similarly, C-mannosylation of extracellular matrix proteins (thrombospondin-1 and –2), axonal guidance proteins, F-spondin, M-spondin, SCO-spondin, semaphorins F and G, RNase 2, proteins involved in angiogenesis (e.g., brain-specific angiogenin inhibitors BAI-1, -2 and –3), metalloproteases (ADAMTS), aggrecanase, GON-1, procollagen I N-proteinase, lacunin, TRAP, and cytokine receptors (e.g., receptors for erythropoietin, growth hormone, prolactin, Interleukin-2, -3, -4, -5, -6, -7, -9, -11, -13, GM-CSF, G-CSF, leukaemia inhibitory factor, oncostatin-M, ciliary neurotrophic factor, thrombopoieitin, calcitonin and leptin) can also be modulated to achieve the desired effect. Thus, modulators of CMT activity are useful in modulating cell proliferation, signal transduction, neuroregeneration and neurodegneration, angiogenesis, apoptosis, immunity inflammation, cell trafficking, protein synthesis and transport, and parasite-host interactions.

CMT modulators (activators or inhibitors) may be formulated according to conventional methodology, depending on the exact nature of the modulator, and will typically comprise the modulator or a precursor thereof in association with a biologically acceptable carrier. In considering various therapies, it is understood that such therapies may be targeted to tissues demonstrated to express CMT.

Delivery of the modulator to the affected cells and tissues can be accomplished using appropriate packaging or administration systems. For example, the modulator may be formulated for therapeutic use with agents acceptable for pharmaceutical administration and delivered to the subject by acceptable routes to produce a desired physiological effect. An effective amount is that amount that produces the desired physiological effect.

The invention also provides a specific inhibitor of CMT activity for the manufacture of a medicament for the treatment or prophylactic treatment of diseases or conditions dependent on CMT activity.

signal sequence from eosinophil derived neurotoxin (EDN; shown in bold) (Newton, D.L. et al., 1994, J. Biol. Chem., 269: 26739-26745), annealed to the N-terminal end of GST in pGEX-2T (Pharmacia) and a reverse primer 5'-ATATGAATTCTCAGTCAGTCAAGCCCAT TTAGCCCAAGCGGATCCACGCGGAACC-3' encoding the CMT substrate site AWAKWA, annealed to the thrombin sequence at the C-terminal end of GST in pGEX-2T. The PCR product was subcloned into the Hind III and EcoR I sites of pSMCi (Asselbergs, F.A. & Grand, P., 1993, Anal. Biochem., 209: 327-331) to yield pSMCi-ssEDN-GST-AWAKWA. A number of different CMT substrates were generated by site-directed mutagenesis using degenerate primers. One of these constructs encoded the protein GST-AWAQWA, which was found to be efficiently C-mannosylated (90%) by CMT following expression in HEK-293 cells and was recognised by the modification specific antibody α5-10 (Krieg, J. et al., 1997, J. Biol. Chem., 272: 26687-26692). This construct was used as a template for generating pSMCi-APN-GST-AWAQWA, encoding the membrane bound form of this CMT substrate.

Cell surface bound APN-GST-AWAQWA

A cell surface form of GST-AWAQWA was made by ligating the cDNA encoding the N-terminal membrane-anchor domain of human aminopeptidase N (APN) to the cDNA encoding GST-AWAQWA via an Spe I restriction site and subcloning into the mammalian expression vector pSMCi using the Hind III/ EcoR I restriction sites.

The N-terminal portion of the fusion protein comprising the cytoplasmic-, the transmembrane- and the stalk region of human aminopeptidase N (APN, 204 bp) was cloned out by PCR from the template pTEJ4-humanAPN (Vogel, L.K. et al., 1992, J. Biol. Chem., 267: 2794-2797) using the forward primer 5'-GGGGTACCAGATCTAAGCTTGCCACCAT

GTGAGAATTTGTATTTTCAGGGTACTAGTTCCCCTATACTAGGTTATTGGAAA
ATTAAGGGCCTTGTGCAACCCACTCGACTTCTTTTGGAATATCTTGAAGAAA
AATATGAAGAGCATTTGTATGAGCGCGATGAAGGTGATAAATGGCGAAACA
AAAAGTTTGAATTGGGTTTGGAGTTTCCCAATCTTCCTTATTATATTGATGGT
GATGTTAAATTAACACAGTCTATGGCCATCATACGTTATATAGCTGACAAGC
ACAACATGTTGGGTGGTTGTCCAAAAGAGCGTGCAGAGATTTCAATGCTTG
AAGGAGCGGTTTTGGATATTAGATACGGTGTTTCGAGAATTGCATATAGTAA
AGACTTTGAAACTCTCAAAGTTGATTTTCTTAGCAAGCTACCTGAAATGCTGA
AAATGTTCGAAGATCGTTTATGTCATAAAACATATTTAAATGGTGATCATGTA
ACCCATCCTGACTTCATGTTGTATGACGCTCTTGATGTTGTTTTATACATGGA
CCCAATGTGCCTGGATGCGTTCCCAAAATTAGTTTGTTTTAAAAAACGTATTG
AAGCTATCCCACAAATTGATAAGTACTTGAAATCCAGCAAGTATATAGCATG
GCCTTTGCAGGGCTGGCAAGCCACGTTTGGTGGTGGCGACCATCCTCCAAA
ATCGGATCTGGTTCCGCGTGGATCCGCTTGGGCTCAGTGGGCT

The translated sequence encoded by the cDNA (308 aa) is:

MAKGFYISKSLGILGILLGVAAVCTIIALSVVYSQEKNKNANSSPVASTTPSASAT
TNPASATTLDQSENLYFQGTSSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYER
DEGDKWRNKKFELGLEFPNLPYYIDGDVKLTQSMAIIRYIADKHNMLGGCPKER
AEISMLEGAVLDIRYGVSRIAYSKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLN
GDHVTHPDFMLYDALDVVLYMDPMCLDAFPKLVCFKKRIEAIPQIDKYLKSSKYI
AWPLQGWQATFGGGDHPPKSDLVPRGSAWAQWA

Within the translated sequence, KSLGILGILLGVAAVCTIIALSVVYSQ is the transmembrane region of APN;
EKNKNANSSPVASTTPSASATTNPASATTLDQS is the stalk domain of APN;

ENLYFQG is the TEV cleavage site;

LVPRGS is the thrombin cleavage site; and

AWAQWA is the CMT substrate.

buffer for 30 min at 4°C. The stained cells were washed and resuspended in 500 µl ice-cold PBS and analyzed on a Becton Dickinson FacsCalibur using CellQuest software. Negative controls consisted of transfected cells probed with normal rabbit IgG or untransfected cells incubated with specific antibodies.The anti-GST antibodies showed that approximately 50% of the cells expressed the GST-CMT substrate fusion protein on their surface. The majority of this protein was C-mannosylated as shown by stainig with the α5-10 antibodies. The negative controls showed low background staining. Thus, C-mannosylation of a cell-surface expressed CMT substrate has been established for the first time, allowing a simple format for cell-based assays.

Example 2:Cell-based assays for CMT activity

The system is exemplified here using the surface-associated marker protein containing a C-mannosylation motif that, when modified, can be recognized by protein chemistry analysis. The methodology was essentially as described in Example 1 but protein chemistry analysis replaces or complements the flow cytometry step used above.

Protein chemistry analysis

The expressed CMT substrates were purified from transfected cells and analyzed by LC-MS for the presence of C-mannosylated peptides essentially as follows. Approximately 5 x 10^7 cells were transfected with pSMCi-APN-GST-AWAQWA cDNA and harvested as described above. After washing the cells in ice-cold PBS, the cell pellet was resuspended in a final volume of 500 µl TEV buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 5 mM DTT). One hundred units of TEV protease (Invitrogen) was added and mixed gently for 7 hours at 16°C. FACS analysis showed that more than 70% of the GST-AWAQWA protein was released from the cell surface after 5 hours of incubation.

one of skill in the art that other methods can be easily devised for high-through put formats for identifying the presence of the modified CMT substrate released from the cell surface, using detectable labels or antibodies, for example.

Example 3 Cell-based assay for CMT activity

In this example, instead of using a protease to release the CMT substrate from the cell substrate, a secreted form of the CMT substrate is used. The GST-AWAQWA fusion protein was obtained from the conditioned medium of cells transfected with pSMCi-ssEDN-GST-AWAQWA described in Example 1. The conditioned medium was concentrated 35-fold by ultrafiltration using a Centricon plus-80 tube (Millipore). GST fusion protein purification and subsequent peptide release is performed as described in Example 2.

Example 4 Cell-based screen for inhibitors of CMT

The assay described in Example 1 can be used for efficient screening of agents as inhibitors of CMT. Inhibitory agents can be detected by the absence or reduction of antibody binding to the cells. In addition, C-mannosylation requires DPM. A second reporter molecule, such as CD59, containing a glycosylphosphatidylinositol (GPI) anchor can optionally be included, serving as a positive marker to exclude inhibitors of the DPM synthesis pathway.

In brief, human embryonic kidney cells (HEK293T) cells that stably express APN-GST-WAQWA and optionally CD59 are seeded into the wells of a 384-well microtiter plate at approximately 20% confluency. A test agent or combinations of test agents can be added to a final concentration of 1 mM from 10 mM stock solutions in DMSO or other suitable concentrations or solvents, as will be apparent to one of ordinary skill in the art. After addition of the agents cells are grown to approximately 90% confluency, washed and stained with α5-10

What is claimed is:

1. A method of assaying for C-mannosyltransferase (CMT) activity, said method comprising the steps of:
   i) providing CMT and a CMT substrate,
   ii) providing conditions conducive to forming a C-mannosylated CMT substrate by action of said CMT on said CMT substrate;
   iii) immobilizing said C-mannosylated CMT substrate and
   iv) detecting said C-mannosylated CMT substrate.

2. The method according to claim 1, wherein said immobilizing step (iii) comprises expressing a cell surface bound CMT substrate on a cell.

3. The method according to claim 1 or claim 2, wherein said C-mannosylation of said substrate is detected using an antibody.

4. The method according to claim 3, wherein said antibody is specific for C-mannosylated CMT substrate.

5. The method according to claim 1 or 2, wherein said C-mannosylation of said substrate is detected using a label.

6. The method according to any one of the preceding claims, wherein a fusion protein comprises the C-mannosylated substrate.

7. The method according to claim 6, further comprising cleaving said fusion protein with a protease.

8. A method of identifying an agent effective in modulating C-mannosyltransferase (CMT) activity, said method comprising the steps of

17. The method of any one claims 9 to 16, said method further comprising detecting the presence of a GPI anchor.

18. A method of identifying an agent effective in modulating C-mannosyltransferase (CMT), said method comprising the steps of:

(a) providing a non-human wild-type or genetically engineered animal comprising a CMT gene;

(b) administering an agent to the non-human transgenic animal; and

(c) determining whether CMT activity is affected relative to when said agent is absent.

19. An agent identified by any one of claims 9 to 18.